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Dealing with hydrocarbons: from Planctomycetes to microbial community

Teresa Filipa Botelho Carvalho

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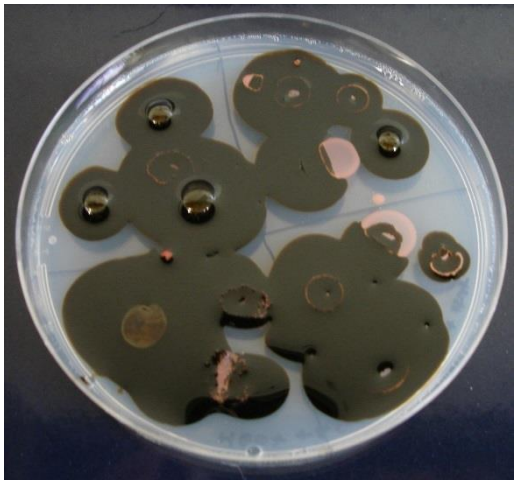
Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Ecologia e Ambiente

2017



Dealing with Hydrocarbons: from Planctomycetes to microbial community

Teresa Filipa Botelho Carvalho
Mestrado em Ecologia e Ambiente
Departamento de Biologia
2017

Orientador
Olga Maria Oliveira da Silva Lage, Professor auxiliar, Faculdade de Ciências da Universidade do Porto

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

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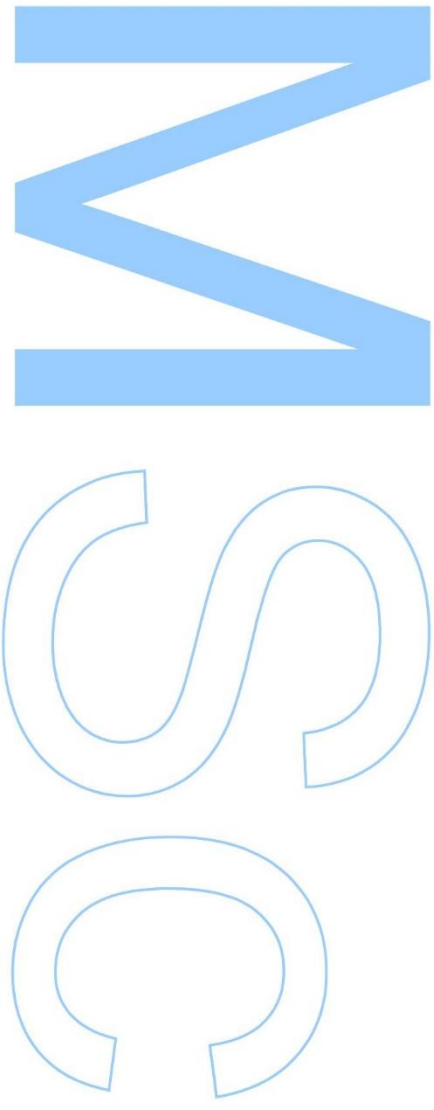


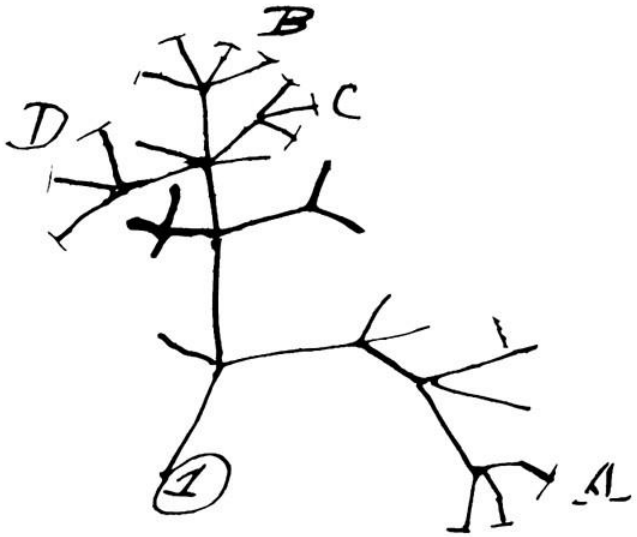
Dissertação para a candidatura ao grau de Mestre em Ecologia e Ambiente submetida à Faculdade de Ciências da Universidade do Porto.

O presente trabalho foi desenvolvido sob a orientação científica da Professora Doutora Olga Maria Oliveira da Silva Lage e foi realizado na Faculdade de Ciências da Universidade do Porto.

Dissertation to apply for the Master's Degree in Ecology and Environmental, submitted to the Faculty of Sciences of the University of Porto.

The present work was developed under the scientific supervision of Professor Dr. Olga Maria Oliveira da Silva Lage and was conducted at the Faculty of Sciences of the University of Porto.

I think



"It is not the strongest of the species that survives, not the most intelligent that survives. It is the one that is the most adaptable to change."

Charles Darwin

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Resumo

Os hidrocarbonetos são uma das potenciais ameaças ambientais que enfrentamos atualmente e que precisa de uma solução urgente. A bioremediação realizada por microrganismos está atualmente a tornar-se numa das principais técnicas, que de forma eficiente e de baixo custo económico, é utilizada para resolver problemas de poluição com hidrocarbonetos. O estudo realizado ao longo do desenvolvimento desta tese tem como objetivo aprofundar o conhecimento das comunidades bacterianas associadas a ambientes ricos em hidrocarbonetos e em particular avaliar a capacidade dos planctomycetes como biodegradadores de hidrocarbonetos. Alguns problemas apresentados por uma companhia de petróleo, Galp energia, SGPS, S.A, foram também comtemplados neste trabalho. Foram desenvolvidas várias experiências de crescimento bacteriano onde os planctomycetes foram cultivados em diferentes meios de cultura e onde os hidrocarbonetos (petróleo bruto, petróleo, óleo de turbina, óleo 150SN, óleo 500SN, gasolina e gasóleo) foram usados como a única fonte de carbono disponível tendo sido demonstrado crescimento efetivo, especialmente em meio de cultura sólido. Além disso, diversos planctomycetes foram capazes de crescer em águas ricas em hidrocarbonetos provenientes de diferentes tanques de tratamento da mesma ETAR. Ambos estes estudos parecem demonstrar a capacidade dos planctomycetes para serem usados na remediação por hidrocarbonetos. Ensaios agudos de toxicidade revelaram que os planctomycetes não são capazes de sobreviver ao efeito tóxico do furfurol, uma substância que causa imensos problemas nas ETARS desta refinaria. A comunidade microbiana presente nas lamas da ETAR, amostra também gentilmente cedida pela Galp, foi isolada e caracterizada filogeneticamente. Foi obtido um total de 117 isolados mas apenas 74 desses isolados foram caracterizados. Os isolados pertencem a diversos grupos taxonómicos, *Firmicutes* (42%), *Proteobacteria* (40%), *Actinobacteria* (16%) e *Bacteroidetes* (1%), e a diversos géneros, sendo que os mais abundantes foram *Bacillus*, *Exiguabacterium*, *Arthobacter* e *Pseudomonas.* Muitos destes isolados são comuns em ambientes poluídos em hidrocarbonetos. Seis desses isolados (uma *Actinobacteria*, uma *Gammaproteobacteria* e quatro *Alfaproteobacteria*) apresentam uma baixa similaridade do gene 16S rRNA relativamente à espécie mais próxima descrita (menos de 96.3%) o que faz destes isolados potenciais novas espécies. Este trabalho mostra, através de diferentes abordagens, o potencial dos Planctomycetes como degradadores de hidrocarbonetos o que faz destes organismos bons candidatos para processos de remediação de ambientes poluídos por

hidrocarbonetos. Este estudo também contribuiu para o alargamento do conhecimento da comunidade microbiana associada a este tipo de habitats.

Palavras-chave: Hidrocarbonetos, Planctomycetes, Comunidade Bacteriana, Lamas e Furfurol.

Abstract

Hydrocarbons are a main environmental problem that needs urgent solutions. Bioremediation by microorganism is an important way to overcome hydrocarbon pollution in a very efficient and low cost methodology. The study reported in this Thesis aimed to in-depth our knowledge on microbial communities of hydrocarbon environments and in particular addressing planctomycetes as potential hydrocarbon degraders. Problems faced by an oil company, Galp energia, SGPS, S.A, were also contemplated in this work. Several experiments where planctomycetes were cultivated in different media and where hydrocarbons (crude oil, fuel oil, turbine oil, 150SN oil, 500SN oil, gasoline and diesel) were used as the only carbon source, was done and these results showed, especially in solid media, effective growth of these bacteria. Furthermore, planctomycetes were able to growth in water from these tanks rich in hydrocarbons. Both these studies seem to indicate the potential of planctomycetes to be used in hydrocarbon scavenging. An acute toxicity assay revealed that planctomycetes are not able to survive to the toxicity effect of furfural, a substance that induces problems in waste water treatment tanks of the oil company. The microbial community from sludge of a wastewater treatment tank, also kindly provided by Galp, was isolated and phylogenetically characterized. A total of 117 isolates were obtained but only 74 were characterized in due time. These belong to *Firmicutes* (42%), *Proteobacteria* (40%), *Actinobacteria* (16%) and *Bacteroidetes* (1%) and the most abundant genera were *Bacillus*, *Exiguabacterium*, *Arthrobacter* and *Pseudomonas*. Many of the isolates are commonly found in hydrocarbon polluted environments. Six isolates (one *Actinobacteria*, one *Gammaproteobacteria* and four *Alfaproteobacteria*) presented low 16S rRNA gene similarity with the closest described species (less than 96.3%) which make them potential new taxa. This work showed, through different approaches, the potential of Planctomycetes as hydrocarbon degraders which make them good candidates for the remediation of hydrocarbon polluted environments. It also contributed to enlarge our knowledge on the microbial community associated with these habitats.

Key words: Hydrocarbons, Planctomycetes, Microbial community, Sludge, Furfural.

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Abbreviations

%	Percent sign
®	Registered trademark
º C	Degree Celsius
µL	Microliter
Abs	Absorbance at 600mm
C	Carbon
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FF	Fossil fuels
GCC	Global climate change
GHG	Greenhouses gases
Gton	Gigatons
GW	Global Warming
HC	Hydrocarbons
M	Molar
mL	Milliliter
mL/L	Milliliter per Liter
NA	Nutrient Agar medium
NH_4^+	Ammonium
NO_3^-	Nitrate
nm	Nanometers
OTUs	Operational taxonomic units
P	Phosphorus
PCR	Polymerase Chain Reaction
PVC	Planctomycetes, Verrucomicrobia, Chlamydiae group
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
sp.	Specie
spp.	Species
TAE	Tris-Acetate EDTA
™	Trademark symbol
WWT	Waste Water Treatment

1. Introduction

1.1 The problems of using fossil fuels as energy source

The idea of using natural resources for human development and progress sounds almost amazing but unfortunately several problems arise from their utilization. Fossil fuels (FF), compounds that contain high amounts of carbon (C), are natural non-renewable resources. The production and use of these compounds such as gasoline, oil, diesel and lubricants release to the atmosphere large amounts of carbon dioxide (CO_2), one of the main greenhouse gases (GHG) which brings major environmental and public health problems. Furthermore, these compounds are major contaminants regarding both prevalence and quantity in the environment (Tyagi *et al.*, 2011). In 2004, it was estimated that about 13 terawatts (1 TW = 10^{12} W = 3.2 EJ/year) of energy worldwide are consumed, and approximately 80% of that comes from burning FF (Goldemberg *et al.*, 2004). In 2010, combustion of FF added to the atmosphere about 4.5 gigatons (Gton = 10^9 tons) of C per year, in the form of CO_2 (IPCC, 2014). The dependence on FF poses three big risks for the survival of human society as we now know it. The first risk is that we will deplete FF reserves. The second is that geopolitical contention from competition for decreasing resources will may lead to economic and energy disruptions, political tumult, and war. The third is global climate change (GCC) caused by the net increase in atmospheric CO_2 due to combustion of the FF (Rittmann, 2008). The FF we consume today is a very large amount, CO_2 emissions from FF combustion and industrial processes contributed with about 78% of the total GHG emission increase between 1970 and 2010, with a contribution of similar percentage over the 2000–2010 period (IPCC, 2014). If conservation and its efficiency are effective, renewable substitutes for FF will have a chance to slow or reverse global warming (GW), but only if they can be implemented on a very large scale. Thus, scale must be considered when evaluating the value of any renewable-energy scheme (Rittmann, 2008). So, as we are unable to find viable and cleaner alternatives for energy production, it is urgent to find alternatives to minimize the environmental impact and damage produced by FF which result from spills that occur during their exploration, production, refining, transport, storage and usage (Mei and Yin, 2009; Cameron, 2012; Duarte *et al.*, 2012). Many of the components of petroleum hydrocarbons (HC) are biodegradable, but some of them are recalcitrant whilst others have intermediate biodegradability, depending on their structure and physical state. According to van Hamme *et al.* (2003) the biodegradability of the oil

components generally decreases in the following order: n-alkanes > branched-chain alkanes > branched alkenes > low molecular-weight n-alkyl aromatics > mono aromatics > cyclic alkanes > polycyclic aromatic hydrocarbons (PAHs) > asphaltenes. So, the degradation of petroleum pollution by microorganisms has been studied extensively for some classes of these chemical compounds and it was considered to be an effective and economic method for environmental remediation (Eaton and Chapman, 1992; Solano-Serena *et al.*, 2008; Vandecasteele and Monot, 2008).

1.2 Bioremediation: a vision of the future

One technique of environmental remediation that has been widely studied in recent years is bioremediation (McKew *et al.*, 2007; Dellagnezze *et al*., 2014; Roy *et al.*, 2014). This is a technique that utilizes living organisms to remove or attenuate the concentration of pollutants in locals where environmental contamination occurred. Bioremediation can be done by bacteria, yeasts, filamentous fungi, algae, plants and animals and the uses of all this biological material have been reported in different studies with good bioremediation potential (Bamforth and Singleton, 2005; Fernández *et al.*, 2011). Two different and distinct strategies of bioremediation with microorganisms exist: the biostimulation and the bioaugmentation. In biostimulation the growth of the indigenous microbial community is stimulated for the improvement of the biodegradation process. Normally this process involves nutrients addition, such as nitrogen (NO_3^- or NH_4^+) or phosphorus (P) (Calvo *et al.*, 2009). On the other hand, the bioaugmentation implies the introduction, on contaminated areas, of specific microbial strains or consortia known to be efficient degraders, improving thus the degrading potential in these areas (Dellagnezze *et al.*, 2014). Fortunately, we have many microorganisms with the metabolic machinery to use oil as a source of C and energy, by either aerobic or anaerobic pathways. The bioremediation utilizing microorganisms to degrade oil contaminants is a promising technology because of its efficiency and relative cost-efficiency (Das and Chandran, 2011). Obviously for bioremediation to be successful we have to provide these microorganisms with the best conditions to optimize the rates of growth and therefore the HC degradation. The degradation of petroleum by microorganisms is a complex process and depends on the nature and amount of the HC present. Furthermore, the physical and chemical characteristics of the oil and oil surface area are also important determinants for bioremediation success (Das and Chandran, 2011).

1.3 The microorganisms that make the biodegradation of fossil fuels: "the special ones"

The presence of microorganisms in HC polluted environments has long been known. Since the beginning of commercial oil production, almost 140 years ago, petroleum engineers have faced problems caused by microorganisms due to their presence in reservoirs (Magot *et al.*, 2000). The first extensive microbiological study describing the widespread presence of specific microorganisms in oil-producing wells was published in 1926 (Bastin, 1926). HC in the environment are biodegraded primarily by bacteria, yeast, and fungi (Das and Chandran, 2011) and the specific knowledge on these microorganisms is important to achieve a better biodegradation success. Up-to-now the most rapid and complete degradation of organic pollutants occurred under aerobic conditions (Das and Chandran, 2011). A wide variety of bacteria with enzymatic capabilities to degrade complex mixtures of HC have been detected in samples from oil fields and oil reservoirs (Table 1) (Magot et al., 2000) as well in HC contaminated environments (Table 2) (Ollivier and Magot, 2005) such as, soil (Roy et al., 2014), marine environments (Head, et al., 2006) and freshwaters (Cooney, 1984).

Table 1 - Bacteria isolated from oil fields and reservoirs (adapted from Magot et al., 2000)

Species	Origin	References
Acetoanaerobium romashkovii	Oil reservoir	Davydova-Charakhcl'yan et al. (1992b)
Anaerobaculum thermoterrenum	Oil reservoir	Rees et al. (1997)
Archaeoglobus fulgidus	Oil field	Beeder et al. (1994)
Desulfacinum infernum	Oil field	Rees et al. (1995)
Desulfobacter vibrioformis	Oil field	Lien and Beeder (1997)
Desulfobacterium cetonicum	Oil field	Galushko and Rozanova (1991)
Desulfomicrobium apsheronum	Oil field	Rozanova et al. (1988)
Desulfotomaculum halophilum	Oil field	Tardy-Jacquenod et al. (1998)
Desulfotomaculum kuznetsovii	Oil field	Nazina et al. (1988)
Desulfotomaculum nigrificans	Oil field	Nazina and Rozanova (1978)
Desulfotomaculum thermocisternum	Oil field	Nilsen et al. (1996)
Desulfovibrio gabonensis	Oil field	Tardy-Jacquenod et al. (1996)
Desulfovibrio longus	Oil field	Magot et al. (1992a)
Desulfovibrio vietnamensis	Oil field	Nga et al. (1996)
Dethiosulfovibrio peptidovorans	Oil reservoir	Magot et al. (1997)
Geotoga petraea	Oil reservoir	Davey et al. (1993)
Geotoga subterranea	Oil reservoir	Davey et al. (1993)
Haloanaerobium acetoethylicum	Oil reservoir	Rengpipat et al. (1988)
Haloanaerobium congolense	Oil reservoir	Ravot et al. (1997)

Haloanaerobium salsugo	Oil reservoir	Bhupathiraju et al. (1994)
Methanobacterium bryantti	Oil reservoir	Davydova-Charakhcl'yan et al. (1992a)
Methanobacterium ivanovii	Oil reservoir	Borzenkov et al. (1997)
Methanobacterium thermoaggregans	Oil reservoir	Ng et al. (1989)
Methanobacterium thermoalcaliphilum	Oil reservoir	Davydova-Charakhcl'yan et al. (1992a)
Methanobacterium thermoautrophicum	Oil reservoir	Olliver and Magot unpub.
Methanocalculus halotolerans	Oil reservoir	Ollivier et al. (1998)
Methanococcus thermolithotrophicus	Oil reservoir	Nilsen and Torsvik (1996)
Methanohalophilus euhalobius	Oil reservoir	Obraztsova et al. (1988)
Methanoplanus petrolearius	Oil reservoir	Ollivier et al. 1997
Methanosarcina mazei	Oil reservoir	Borzenkov et al. (1997)
Methanosarcina sicíliae	Oil reservoir	Ni and Boone (1991)
Petrotoga miotherma	Oil reservoir	Davey et al. (1993)
Spirochaeta smaragdinae	Oil reservoir	Magot et al. (1992b)
Thermoanaerobacter brockii	Oil reservoir	Cayol et al. (1995)
Thermodesulfobacterium mobile	Oil field	Rozanova and Pivovarova (1988)
Thermodesulforhabdus norvegicus	Oil field	Beeder et al. (1995)
Thermotoga elfii	Oil reservoir	Ravot et al. (1995)
Thermotoga hypogea	Oil reservoir	Fardeau et al. (1997)
Thermotoga subterranea	Oil reservoir	Jeanthon et al. (1995)

Table 2 - Genera of bacteria able to grow using HC as unique source of C and energy isolated from HC polluted environments. Adapted from Ollivier and Magot, 2005

Genus	Typical substrate	References
Achromobacter	Gas oil	Le Petit et al. (1975)
Acidocella	Naphtalene	Dore et al. (2003)
Acidovorax	Phenanthrene	Meyer et al. (1999)
Acinetobacter	Gas oil	Le Petit et al. (1975)
Actynomyces	Crude oil	ZoBell (1946)
Aeromonas	Diesel oil	Odokumal and Dickson (2003)
Agrobacterium	Gasoline aromatics	Prantera et al. (2002)
Alcaligenes	Gas oil	Le Petit et al. (1975)
Alcanivorax	Alkanes	Yakimov et al. (1998)
Alkanindiges	Alkanes	Bogan et al. (2003)
Alteromonas	Crude oil	Iwabuchi et al. (2002)
Arthrobacter	Gas oil	Le Petit et al. (1975)
Aureobacterium	Crude oil	Giles et al. (2001)
Azoarcus	Toluene	Hess et al. (1997)
Azospirillum	Jet fuel	Eckford et al. (2002)
Azotobacter	Crude oil	Gradova et al. (2003)
Bacillus	Toluene	ZoBell (1946)
Beijerinckia	Phenanthrene	Surovtseva et al. (1999)
Blastochloris	Toluene	Zengler et al. (1992)

Brevibacterium	Alkanes	Pirnik et al. (1974)
Brevundimonas	Fuel Oil	Chaineau et al. (1992)
Burkholderia	Toluene	Parales et al. (2000)
Clavibacter	Naphtalene	Dore et al. (2003)
Comamonas	Phenanthrene	Meyer et al. (1999)
Corynebacterium	Fuel Oil	Chaineau et al. (1992)
Cycloclasticus	Biphenyl	Dyksterhouse et al. (1995)
Cytophaga	Crude oil	Bossert and Bartha (1984)
Dechloromonas	Benzene	Coates et al. (2001b)
Desulfatibacillum	Alkanes	Cravo-Laureau et al. (2004)
Desulfobacterium	Xylene	Harms et al. (1999)
Desulfobacula	Toluene	Rabus et al. (1993)
Desulfosarcina	Xylene	Harms et al. (1999)
Desulfosporosinus	Gasoline	Robertson et al. (2001)
Dietzia	Alkanes	Yumoto et al. (2002)
Enterobacter	Alkanes	Saadoun et al. (1999)
Envinia	Alkanes	Saadoun et al. (1999)
Flavobacterium	Diesel oil	Stucki and Alexander (1987)
Geobacillus	Crude oil	Nazina et al. (2001)
Geobacter	Toluene	Coates et al. (2001a)
Gordonia	Alkanes	Kummer et al. (1999)
Klebsiella	Crude oil	Odokumal and Dickson (2003)
Lactobacillus	Crude oil	Floodgate (1984)
Leclercia	Pyrene	Sarma et al (2004b)
Leucothrix	Crude oil	Floodgate (1984)
Lutibacterium	Phenanthrene	Chung and King (2001)
Marinobacter	Crude oil	Gauthier et al. (1992)
Micrococcus	Hexadecane	Ilori et al. (2000)
Moraxella	Byphenyl	Stucki and Alexander (1987)
Mycobacterium	Phenanthrene	Willumsen et al. (2001)
Neptunomonas	Naphtalene	Hedlund et al. (1999)
Nocardia	Alkanes	Mikolasch et al. (2003)
Nocardioides	Phenanthrene	Iwabuchi and Harayama (1998)
Ochrobactrum	Diesel	Gaylarde et al. (1999)
Oleiphilus	Alkanes	Golyshin et al. (2002)
Oleispira	Alkanes	Yakimov et al. (2003)
Paenibacillus	Phenanthrene	Meyer et al. (1999)
Pasteurella	Fluoranthene	Sepic et al. (1997)
Peptococcus	Alkanes	Floodgate (1984)
Planococcus	Alkanes	Engelhardt et al. (2001)
Polaromonas	Naphtalene	Jeon et al. (2004)
Proteus	Crude oil	Odokumal and Dickson (2003)
Pseudomonas	Gas oil	Le Petit et al. (1975)
Ralstonia	Toluene	Parales et al. (2000)

Rhodococcus	Phenanthrene	Meyer et al. (1999)
Sarcina	Crude oil	Bossert and Bartha (1984)
Serratia	Crude oil	Odokumal and Dickson (2003)
Sphaerotilus	Crude oil	Austin et al. (1977)
Shingomonas	Toluene	Romine et al. (1999)
Spirillum	Crude oil	Bossert and Bartha (1984)
Staphylococcus	Diesel	Saadoun et al. (1999)
Stenotrophomonas	Pyrene	Juhasz et al. (2000)
Streptomyces	Alkanes	Barabas et al. (2001)
Thalassolituus	Alkanes	Yakimov et al. (2004)
Thauera	Toluene	Shinoda et al. (2004)
Thermoleophilum	Alkanes	Zarilla and Perry (1984)
Thermus	Pyrene	Feitkenhauer et al. (2003)
Vibrio	Phenanthrene	Hedlund and Staley (2001)
Xanthobacter	Dibenzothiophene	Padden et al. (1997)
Xanthomonas	Phenanthrene	Hamann et al. (1999)

When microorganisms are exposed to a HC its degradation is initiated with their intracellular attack by an oxidative process, where oxygen is incorporation through enzymatic key reaction catalyzed by oxygenases and peroxidases. The peripheral degradation pathways convert organic pollutants, step by step, into intermediates of the central metabolism. Biosynthesis of cell biomass occurs from the central precursor metabolites and sugars required for various biosynthesis and growth is based on gluconeogenesis (Fig. 1) (Das and Chandran, 2011). The degradation of HC can be mediated by specific enzyme systems such as cytochrome P450 alkane hydroxylases that constitute a super family of ubiquitous monooxygenases which play an important role in the microbial degradation of several HC compounds (van Beilen nd Funhoff, 2007) (Table 3). Higher eukaryotes generally contain several different P450 families that consist of large number of individual P450 forms that may contribute as an ensemble of isoforms to the metabolic conversion of a given substrate. In microorganisms such P450, multiplicity can only be found in few species (Zimmer, *et al.*, 1996)**.** Cytochrome P450 enzyme systems are found to be involved in biodegradation of HC (Table 3) (Das and Chandran, 2011). The diversity of alkane oxygenase systems in prokaryotes and eukaryotes that are actively participating in the degradation of alkanes under aerobic conditions are, for instances, cytochrome P450 enzymes, integral membrane di-iron alkane hydroxylases (*e.g*. alkB), soluble di-iron methane monooxygenases, and membrane bound copper containing methane monooxygenases (Das and Chandran, 2011).

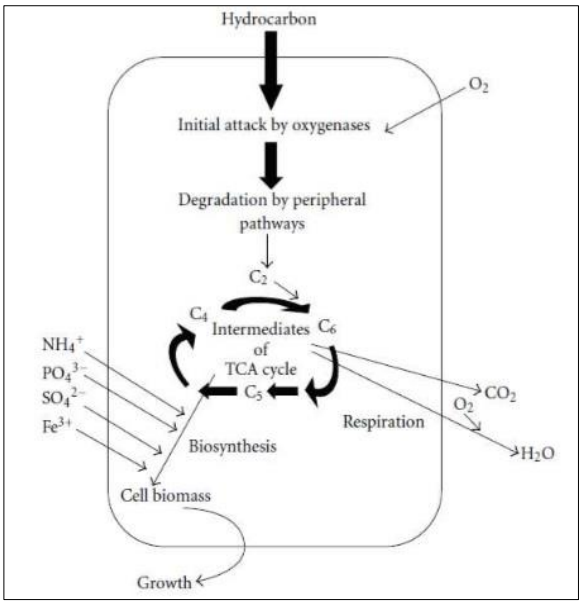

Figure 1 - Main principle of aerobic degradation of HC by microorganisms. Image from Das and Chandran, 2011

Table 3 – Enzymes involved in biodegradation of petroleum HC. Table adapted from Das and Chandran, 2011

Enzymes	Substrates	Microrganisms	References
Soluble Methane Monooxygenases	C_1-C_4 alkanes, alkenes and cycloalkanes	*Methylococcus Methylosinus Methylocystis Methylomonas Methylocella*	McDonald et al. (2006)
Particulate Methane	C_1-C_5 (halogenated) alkanes and cycloalkanes	*Methylobacter Methylococcus Methylocystis*	McDnald et al. (2006)
AlkB related Alkane Hydroxylases	C_5-C_{16} alkanes, fatty acids, alkyl, benzenes, cycloalkanes and so forth	*Pseudomonas Burkholderia Rhodococcus Mycobacterium*	Jan et al. (2002)
Eukaryotic P450	C_{10}-C_{16} alkanes, fatty acids	*Candida maltosa Candida tropicalis Yarrowia lipolytica*	Iida et al. (2000)
alBacterial P450 oxygenase system	C_5-C_{16} alkanes, cycloalkanes	*Acinetobacter Caulobacter Mycobacterium*	van Beilen et al. (2006)
Dioxygenases	C_{10}-C_{30} alkanes	*Acinetobacter sp.*	Maeng et al. (1996)

The characterization of bacterial communities living in this type of environments and the evaluation of their HC degradation capacities could potentially serve as guideline for bioremediation processes in this type of pollution. Furthermore, a lot of microorganism possess other adaptation strategies such as the ability (1) to modify the cellular membrane to maintain the necessary biological functions (de Carvalho and da Fonseca, 2005; Khomenkov et al., 2008; van Der Geize and Dijkhuizen, 2004), (2) to produce surface active compounds as biosurfactants (Ron and Rosenberg, 2002) and (3) to use efflux pumps to decrease the concentration of toxic compounds inside the cells (van Hamme *et al.*, 2003). All these metabolic capabilities make microorganisms an interesting and sound tool for the bioremediation of contaminated sites. Several bacterial strains such as, *Pseudomonas*, *Mycobacterium*, and *Rhodococcus* have been reported to possess the metabolic routes required for the degradation of compounds, like benzene, toluene, ethylbenzene and xylene as well as polyaromatic hydrocarbons such as naphthalene, phenanthrene, anthracene, pyrene and the highly carcinogenic benzo[a]pyrene (Farhadian *et al.*, 2008; Haritash and Kaushik, 2009; de Carvalho *et al.*, 2005).

1.4 Why Planctomycetes?

The *Planctomycetes* are a peculiar group of the domain *Bacteria* that belong to the monophyletic *Planctomycetes, Verrucomicrobia, Chlamydiae* (PVC) superphylum, togheter with *Lentisphaera*, and the *Candidate* groups Poribacteria and OP3 (Wagner and Horn, 2006). They possess a peculiar and unique morphological, genetic, metabolic and physiological identity. The phylum *Planctomycetes* has two classes: the *Phycisphaerae* and the *Planctomycetacia*. The first one, have two orders: the order *Tepidisphaerales* with one genus and one species described and the order *Phycisphaerales* with two genera and two species. The second one, the class *Planctomycetacia* which includes candidate species and two orders: the *Planctomycetales* which comprise seventeen genera with twenty eight species described and the order *Brocadiales* with five genera that has seventeen species described (Uhrig, 2017). Until recently it was thought that they had characteristics that truly differentiate them from other bacteria and prokaryotic organisms, such as, proteic cell walls without peptidoglycan, budding reproduction (Fig. 2), with exception of the *Phycisphaerae* and Anammox planctomycetes, which use binary fission (Fuerst and Sagulenko, 2011; van Teeseling et al., 2015), compartmentalized cell plan and the presence of unusual coat proteins that demonstrate a similarity between the compartmentalization machinery of prokaryotes and eukaryotes (Santarella-Mellwig et al., 2010). All this characteristics led to an increasing interest on these organisms, over the last decades. But Jeske *et al.*, (2015) and van Teeseling *et al.*, (2015) demonstrated that planctomycetes are not an exception to the universal presence of peptidoglycan in the bacterial cell wall because they proved, independently, that two species of planctomycetes, *Planctomyces limnophilus* and *Kuenenia stuttagertensis,* respectively, possessed peptidoglycan in their cell wall in a Gram-negative wall structure. This subject raised a great curiosity to study more and better this phylum that continues still to be very much unknown and with much to be discovered. The Planctomycetes are a ubiquitous group of bacteria which are present in many ecosystems ranging from various aquatic to the terrestrial ones (Fuerst, 1995) and besides, they can also live in association with different eukaryotic hosts like macroalgae (Lage and Bondoso, 2011), ants (Eilmus and Heil, 2009), ascidians (Da Silva Oliveira *et al.*, 2013), invertebrates (Chaiyapechara *et al.*, 2012), corals (Webster and Bourne, 2007), sponges (Pimentel-Elardo *et al.*, 2003), macrophytes (He *et al.*, 2012), *Sphagnum* peat bogs (Kulichevskaya *et al.*, 2006), lichens (Grube *et al.*, 2012) and within the rhizosphere of several plants (da Rocha *et al.*, 2009) as well as associated to the gut microbiome of humans (Cayrou *et al.*, 2013) and other mammals. The

planctomycetes were described as having an important role in the biogeochemical cycles like carbon (McCarren and DeLong, 2007), nitrogen (Kalyuzhnyi *et al.*, 2010) and sulphur (Wegner *et al.*, 2013). Due to their particular characteristics, they can survive in extreme environments such as hypersaline (Baumgartner *et al.*, 2009), hot springs (Tekere *et al.*, 2011), glacial waters (Zeng *et al.*, 2013), acidophilic habitats (Ivanova and Dedysh, 2012), polluted habitats (Chouari *et al.*, 2003) and even in HC polluted environments (Abed *et al.*, 2007; Abed *et al.*, 2011; Dos Santos *et al.*, 2011; Peng *et al.*, 2015; Conlette, 2016). Guo et al. (2014) showed that several species of Planctomycetes share diverse genomic variations and unique genomic characteristics. Their adaptations to such a wide range of environments and those genomic variations and unique characteristics make Planctomycetes good potential candidates for biotechnological applications. This potential has triggered several studies in recent years, for example, it was shown that the planctomycetes are possibly good candidates for assessing water quality (Flores *et al.*, 2014) and they are able to produced bioactive compounds (Jeske, *et al.*, 2013; Graça, *et al.*, 2016). Furthermore, the potential of planctomycetes as alternative or supplementary food source for *Daphnia* spp., organisms that play a key role in energy transfer in freshwater food webs as a primary consumer was tested (Antunes, *et al.* 2016). These applicabilities associated with the fact that they survive in HC polluted environments and that they have genes responsible for expression products that catalyze carbon-halogen bond cleavage (example: haloalkane dehalogenase genes (drbA) from *Rhodopirellula baltica* SH1) (Jesenská et al., 2009), are indicative of a potential capacity of these bacteria to be used in bioremediation processes.

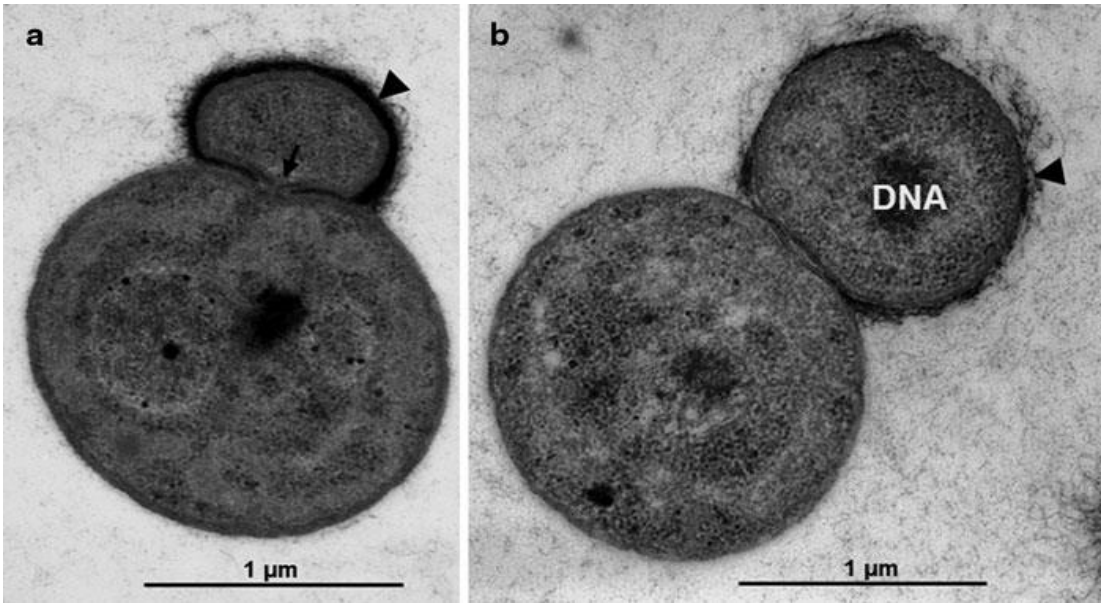

Figure 2 - Planctomycetes: Ultrathin sections of two budding cells of *Aquisphaera giovannonii* by chemical fixation for transmission electron microscopy. Image provided by Olga Lage (Lage, Bondoso, and Lobo-Da-Cunha, 2013)

2. Objectives

Our initial goal was to explore the role of Planctomycetes in the degradation of HC aiming at their potential use in bioremediation processes. However, during a meeting with Galp energia, SGPS, S.A, a big oil company, other research lines popped up and the aims of this thesis were enlarged to address problems faced by this company. Thus, beside the initial goal it was also aimed: (1) to assess if planctomycetes were able to grow in the presence of furfural. Furfural is a compound that way reach the WWT tanks and way affect the existent biodiversity; (2) to assess if planctomycetes were able to grow in water from WWT tanks, strengthening the idea that the planctomycetes are able to degrade HC and could be helpful in the refinery waste treatment; (3) Isolation and phylogenetic characterization of bacteria present in the sludge of WWT tanks from Galp refinery to disclose the bacteria that live in this type of environment and understand more about the processes that happen in the refinery tanks .

3. Materials an Methods

3.1 Biological Material

In this work, several strains of planctomycetes were used. These strains have different origins and belong to different species. *Rhodopirellula baltica* strain SH1 was isolated from the Baltic sea (Schlesner, 1994), *Rhodopirellula rubra* strains LF2, UC9, FC3 and MsF5, *Roseimaritima ulvae* strain UC8, *Mariniblastus fucicola* strain FC18, *Planctomyces sp*. strain Pd1, *Rubinisphaera brasiliensis* strain Gr7 and a new genus strain FF15, were all isolated from the biofilm community of different marine macroalgae (Lage and Bondoso, 2011), and are part of the LEMUP (Laboratory of Microbial Ecophysiology of University of Porto, Porto, Portugal) microbial collection.

A sample of sludge from a waste water treatment (WWT) tank was kindly provided by Galp, for microorganism isolation. All the biological strains obtained from this sludge will became part of LEMUP microbial collection.

3.2 Media used in this study

Several media for the growth of microorganisms were used in this study. The composition of all media are referred in Table 4. Medium M13 used to grow planctomycetes was prepared according to Lage and Bondoso (2011). With except of nutrient agar (NA) all other media used in this study were adaptations from M13 in which their nitrogen and/or carbon sources were changed.

3.3 Organic compounds/mixtures used in this study

In this work we used several hydrocarbons as unique carbon source for planctomycetes growth: crude oil, fuel oil, turbine oil, and base oils from group I: 150SN and 500SN gently provided by Galp, as well as gasoline and diesel from commercial origin. Samples of wastewaters rich in hydrocarbons from WWT tanks, also provided by Galp, were used for testing the growth capability of planctomycetes in hydrocarbon polluted waters.

Table 4 – Composition of the media used in this study.

	M13	M1G	M1H	M2	M3	M4	M5G	M5H	M6G	M6H	NA
Peptone	0.025%	-	-	-	-	-	-	-	-	-	0.5%
Yeast Extract	0.025%	-	-	-	-	-	-	-	-	-	0.3%
$NH_4(SO_4)_2$	-	0.1%	0.1%	0.2%	-	-	1.0%	1.0%	-		-
$NaNO_3$	-	-	-	-	0.1%	0.2%	-	-	1.0%	1.0%	-
Glucose	0.025%	0.025%	-	0.025%	0.025%	0.025%	0.025%	-	0.025%	-	-
HYDC	-	-	drops	-	-	-	-	1.0 %	-	1.0 %	-
Hutner's salts *	20mL/L	20mL/L	20mL/L	20mL/L	20mL/L	20mL/L	20mL/L	20mL/L	20mL/L	20mL/L	-
Vitamins nº6 **	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	-
Tris-Hcl 0.1M	50mL/L	50mL/L	50mL/L	50mL/L	50mL/L	50mL/L	50mL/L	50mL/L	50mL/L	50mL/L	50mL/L
Distillate water	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	10mL/L	1000mL
Sea water	900mL/L	900mL/L	900mL/L	900mL/L	900mL/L	900mL/L	900mL/L	900mL/L	900mL/L	900mL/L	-
Agar	-/1.6%	-/1.6%	-/1.6%	-	-	-	-	-	-	-	1.6%

* Cohen-Bazire et al. (1957)
** 0.1 µg/mL Cyanocobalamine, 2.0 µg/mL biotin, 5.0 µg/mL thiamine-HCl, 5.0 µg/mL Ca-pantothenate, 2.0 µg/mL folic acid, 5.0 µg/mL riboflavin and 5.0 µg/mL nicotinamid

3.4 Testing Planctomycetes growth with hydrocarbons as unique carbon source

3.4.1 Growth in solid media

To confirm previous results, we tested the growth of several planctomycetes in solid media with different hydrocarbons (HC) as unique carbon source. The HC used in this test were crude oil, fuel oil, 150SN oil, 500SN oil, turbine oil, gasoline and diesel fuel. For this, the 10 different strains of planctomycetes referred above were grown in M13 (with 1.6% agar) and in medium M1G (with 1.6% agar) as a control media and in M1H to test the possibility of planctomycetes to growth on different HC. For that, we grew all the strains in M1H and 3 x 10 µl droplets of each strain were inoculated on top of drops of the various HC. All cultures were grown at 25ºC in the darkness and, daily, checked for the presence of growth.

3.4.2 Growth in liquid media

In an attempt to grow planctomycetes in liquid media with HC_S as unique carbon source, we selected six strains SH1, LF2, UC8, FC18, Gr7, FF15 because they are representatives from different genera and also because of logistics as well as time constrains the number of strains had to be reduced. These strains were grown in several test media (20 mL culture) with different nitrogen sources (NH_4^+ or NO_3^-) at different concentrations (0.1%, 0.2% and 1%). These media were designated M1, M2, M3, M4, M5G and M6G as described in Table 4. Furthermore, media M5H and M6H (with 1% nitrogen source) were also assayed. In these, instead of glucose the carbon sources were two hydrocarbon oils - 150SN and 500SN. Triplicate cultures of the six planctomycetes strains were tested in M13, M5G, M6G, M5H (500SN oil) and M6H (500SN oil). All these cultures were maintained at 25ºC with strong agitation (about 500 rpm and with slight inclination) during seven or twenty one days. The growth at the cultures was quantified by measuring the turbidity of cultures in a spectrophotometer GenesysTM 10 Series (Thermo Spectronic) at 600 nm at different time intervals.

3.5 Planctomycetes acute assay in the presence of furfural

In this assay we wanted to test the resistance of planctomycetes to furfural. This compound is a real big problem in WWT tanks of Galp because when this compound arrives to the treatment plants it affects the microbial consortium present in the sludge and consequently the treatment of the waste waters. So, if Planctomycetes showed resistance to this compound, they might be used on its bioremediation. An acute toxicity assay (30 minutes exposure) was used to assess furfural toxicity in strains FC3, FC18, FF15, Gr7, LF2, MsF5, Pd1, SH1, UC8 and UC9 and after that we checked the cellular viability. For that, we collected the cells from 1 ml culture after centrifugation (MiniSpin®, eppendorf) at 13400 rpm during 60 seconds. The cells were then resuspended in 1 mL of a solution with furfural at different concentrations (10^0, 10^{-1}, 10^{-2} and 10^{-3}) and exposed for 30 minutes. After this time, the suspension was again centrifuged and resuspended in sterile sea water. To checked the viability of the exposed cells, we cultivated them in M13 agar medium using the drop plate method, by inoculating 3 drops of 10 µl from each concentration. Cell growth was checked every day. To classify the growth of the tested strains we used a range from 0 to 4, in which 4 represents utmost growth (positive control) and 0 represents the absence of growth, according to Flores, *et al.* (2014).

3.6 Assessment of Planctomycetes growth in the water from waste water treatment tank of the refinery

In an attempt to test if water rich in HC from WWT tank of Galp refinery was able to support planctomycetes growth, strains LF2 and Gr7 were inoculated in base media M1H medium with the referred water as the only carbon source after 0.2 µm sterilization. For this some drops of the waste water were put on top of the medium and 3 x 10 µl drops of each culture were placed on top of the water drops. The growth of the culture was checked daily. The presence of planctomycetes was confirmed by observation under the optical microscope.

3.7 Isolation and characterization of microorganisms from sludge of waste water treatment tanks of Galp refinery

Sludge from a WWT tank provided by Galp refinery was used for the isolation of the microbial community and posterior taxonomic characterization.

3.7.1 Isolation process

The sample of sludge collected in a waste water treatment tank was maintained at 4ºC until being processed. At first two cryovials containing 1 mL of sample supplemented with 27% glycerol were placed, at -80°C. For the isolation, 100 µl of the sludge sample were inoculated in three different media, NA, M13 agar and M1H agar where 3 different hydrocarbons were used separately as unique carbon sources: crude oil and oils 150SN and 500SN. This three media had different purposes. With NA isolation we tried to isolate freshwater microorganisms while with M13 agar and M1H agar, both marine media, we tried to isolate marine organisms or microorganisms able to degraded HC, respectively. In NA isolation, 100 µl of serial dilutions (10^{-1} to 10^{-4}) were also inoculated. The cultures were incubated in the dark at 26ºC and growth was monitored daily. All colonies with distinct morphology were transferred to the respective media of isolation. Confirmed isolated pure strains were cultivated in liquid media and 1ml of that culture was cryopreserved with 27% glycerol at -80ºC. The bacterial isolates obtained were named according to the isolated media, as described in Table 5.

Table 5 - Designation of the bacterial isolates**.** The isolates were named with codes and numbered, being the numbers represented by #.

Isolate code	Isolation medium
M607_#	M13
NA_#	NA
PET_#	M1H with crude oil as carbon source
150SN_#	M1H with 150SN oil as carbon source
500SN_#	M1H with 500SN oil as carbon source

3.7.2 Identification and characterization of isolates

One mL from all strains grown in liquid medium were centrifuged and cell pellets collected and frozen at -20 ºC. DNA from the frozen cell pellets was extracted using E.Z.N.A. ® Bacterial DNA Kit from OMEGA (bio-tek), according to the commercial protocol. DNA extraction was confirmed by electrophoresis in a 1.2% agarose gel stained with Roti Safe (Roth) in 1 x Tris Acetate and EDTA (TAE) buffer (OMEGA). The taxonomic identification of the isolates was based on the analysis of the 16S rRNA gene. This gene was amplified from the extracted DNA with the universal primers, 27F and 1492R in 25 µl of NZYTaq II 2x Green Master Mix from Nzytech (genes & enzymes) and two µl of DNA from each isolate. The PCR program was performed in a MyCycler™ Thermo Cycler (Bio-Rad) and amplification conditions comprised initial denaturing step of 5 minutes at 95 ºC; 30 cycles of 1 minute at 94 ºC; 1 minute at 52 ºC, 90 seconds at 72 ºC and a final extension of 5 minutes at 72 ºC. PCR products were visualized after electrophoresis in a 1.2% agarose gel stained with Roti Safe (Roth) in TAE buffer. The PCR amplicons were purified with illustra GFX PCR DNA and Gel Band Purification Kit from GE Healthcare and sequenced at GATC Biotech (Constance, Germany). Sequence analyses were carried out using Geneious R10, version 10.2.3, (Biomatters limited, Auckland, New Zealand). The obtained sequences were compared in the Ribosomal Database Project (RDP) to establish their closest relatives. The phylogenetic tree was constructed using Geneious R10 tree builder, by choosing the Tamura-Nei model and the neighbor-joining method with a bootstrap resampling method considering 1000 replicates. The *Archaea Sulfolabus acidocaldarius* was used as outgroup.

4. Results and Discussion

4.1 Testing Planctomycetes growth with hydrocarbons as the unique carbon source

4.1.1 Growth in Solid media

To test the capacity of planctomycetes to metabolize hydrocarbons, M13 agar, M1G agar medium and M1H agar medium with different hydrocarbons as only carbons source were use. With the exception of gasoline and diesel, crude oil, fuel oil, turbine oil, 150SN and 500SN oils were all effective in supporting the growth of the 10 strains of planctomycetes (*Rhodopirellula baltica* strain SH1, *Rhodopirellula rubra* strains LF2, UC9, FC3 and MsF5, *Roseimaritima ulvae* strain UC8, *Mariniblastus fucicola* strain FC18, *Planctomycetes sp.* strain Pd1, *Rubinisphaera brasiliensis* strain Gr7 and a new genus, strain FF15) (Fig. 3). When Planctomycetes were cultivated in medium M1H growth was only visible after 10 to 15 days depending on the strain and hydrocarbon. The results of this assay were confirmed with growth controls, M13 and M1G where growth was visible after 2 to 3 days. This showed that planctomycetes needed a long adaptation phase (lag phase) to switch their metabolism to hydrocarbon utilization. As a follow up of these initial promising hydrocarbons degradations by planctomycetes it was aimed their cultivation in liquid media.

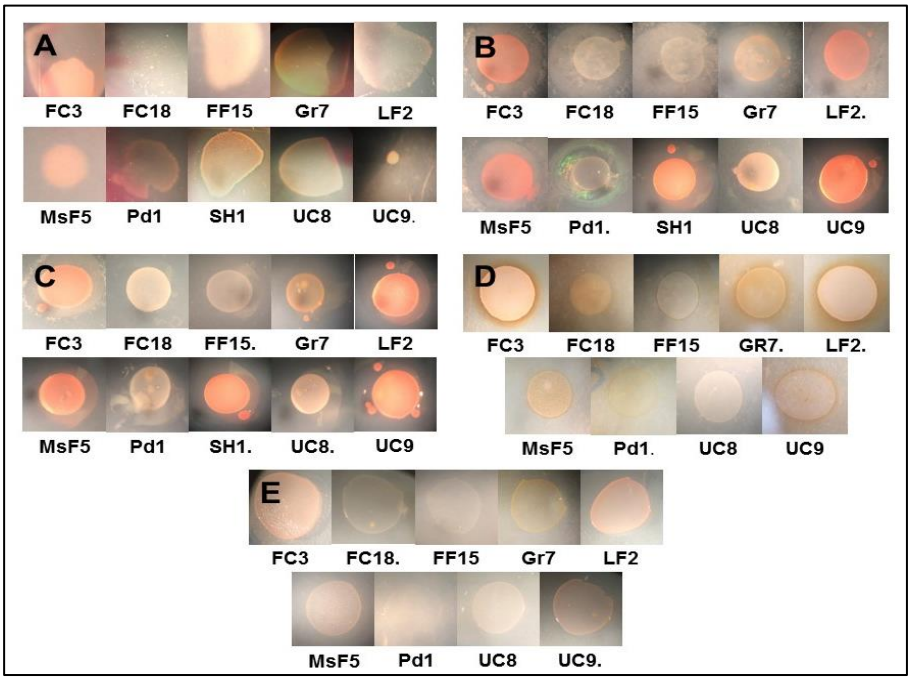

Figure 3 – Growth of all tested strains (specified in the figure) after 21 days exposure to: A – Fuel oil; **B –** Oil 500SN; **C –** Oil 150SN; **D –** Petroleum and **E –** Turbine Oil in media M1H with these HC as the only carbon source

4.1.2 Growth Liquid media

Aiming at developing a medium that allowed the better growth of Planctomycetes in the presence of hydrocarbons, several media with NH_4^+ or NO_3^- as nitrogen source in different concentrations and with glucose as carbon source (M1, M2, M3 and M4) were tested for all the 10 strains of planctomycetes. In general a 24/48h lag phase was observed for all the strains and the most preferred nitrogen source was NH_4^+ or any of the two sources but in a higher concentration (Fig. 4). However, the growth level in these media was lower comparatively to the one in M13 medium that had an organic nitrogen source (peptone and yeast extract).

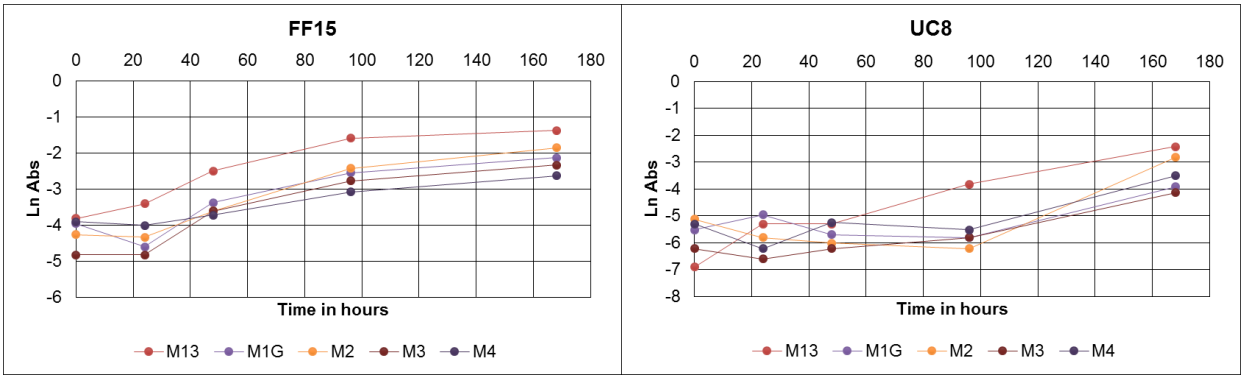

Figure 4 - Growth of strains FF15 and UC8 in different liquid media. Both strains showed the lag phase of 24/48 hours. The strain FF15 preferred both concentrations (0.1% or 0.2%) in same nitrogen source (NH_4^+). The strain UC8 showed that preferred both nitrogen sources (NH_4^+ or NO_3^-) in higher concentrations (M2 and M4, respectively).

Based on these results it was decided to proceed with liquid cultures using NH_4^+ or NO_3^- at an higher concentration (1%). These media were referred as M5G and M6G (when glucose was the carbon source), respectively for NH_4^+ and NO_3^-, and as M5H and M6H when, instead of glucose, possessed one of the two oils 150SN and 500SN as carbon source (Table 4). In general, all the strains tested had a lag phase (approximately of 4 days) and it was observed that after 21 days the planctomycetes presented a good level of growth, comparatively to controls (M13 medium, M5G and M6G) in M5H or M6H with oil 500SN (Fig. 5).

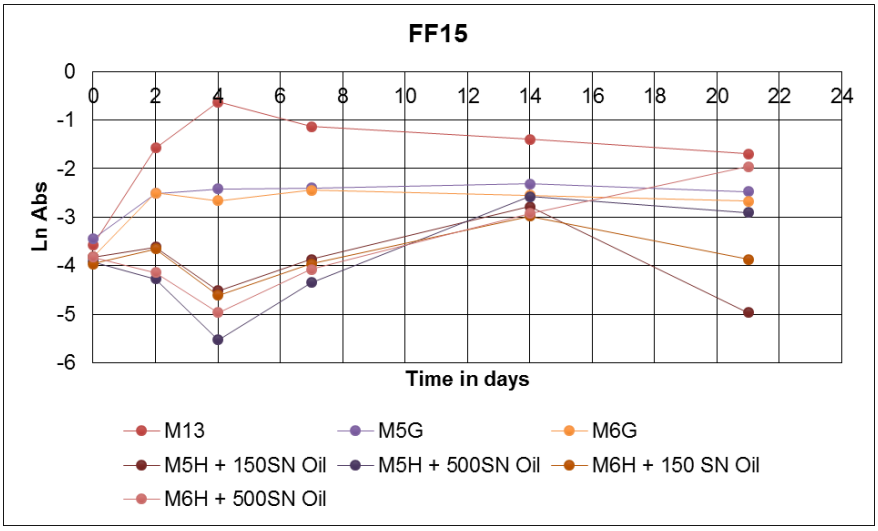

Figure 5 - Growth of strain FF15 with two hydrocarbons (150SN and 500SN oils) as carbon source in two media with different nitrogen source. Strain FF15 showed a 4 days oag phase and the best growth was obtained with 500SN oil after 21 days.

These promising results encouraged us to proceed with a similar assay with cultures in triplicate in M13, M5G, M6G, and M5H as well M6H supplemented with 500SN since it was the oil with better growth. Growth of strains Gr7, LF2, SH1, FC18, FF15 and UC8 in M13 medium showed a normal growth curve, without lag phase and with the stationary phase attained after approximately 2 days (Fig. 6A). The results obtained in M5G and M6G media (Fig. 6B and 6C), showed an initial increase in cell concentration in the first 2 days (although lower than in M13) that was comparable in the two media. After the initial growth, reduction of cell concentration was observed for several strains in a difficult way to be explained. In the presence of 500SN oil as only carbon source, the results were again difficult to interpret. In M5H medium (Fig. 6D) a general initial decrease (lag phase) in 4 strains (UC8, LF2, SH1 and FC18) occurred but after 21 days culture, some of strains showed increased growth (especially FF15 and FC18). In M6H medium (Fig. 6E), although abrupt changes in concentration were observed (especially in strain FF15), the cell concentration varied in a certain range. This up and down values of cell concentration are difficult to understand and explain. The results obtained with liquid cultures do not allow to draw conclusions on the behavior of planctomycetes towards 500SN oil.

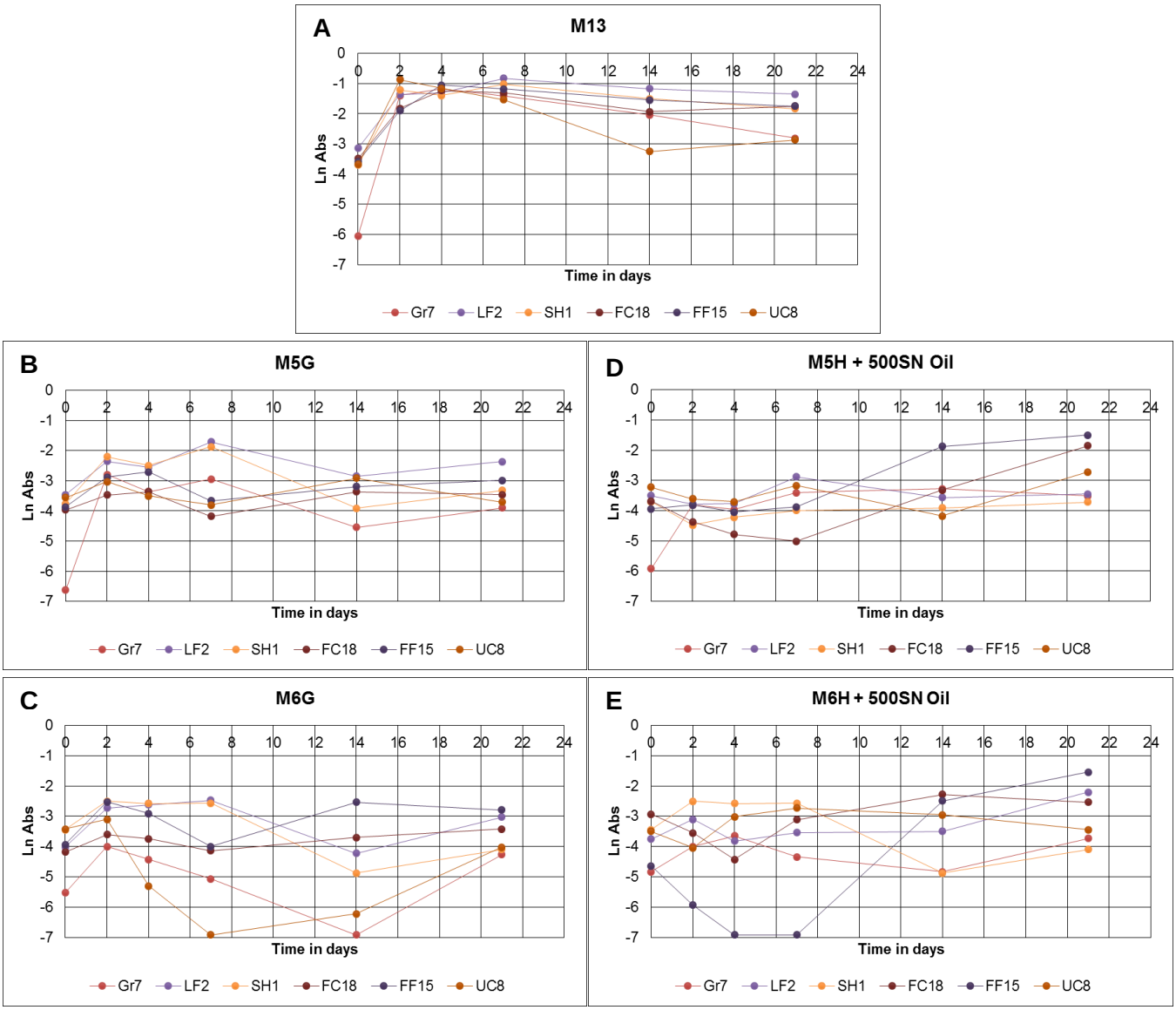

Figure 6 - Growth of six strains of planctomycetes tested in different media with different nitrogen and carbon sources. Normal control growth in M13 medium **(A)**. In M5G **(B)** and M6G **(C)** media the strains showed an increase of cell concentration after 2 days. In M5H **(D)** in general all the strains showed a lag phase but after 21 days, some of the strains showed an increase in growth (FF15 and FC18). In M6H **(E)** the strains showed abrupt changes of the cell concentrations.

Further culturing in liquid media is needed to allow a full comprehension on the behavior of planctomycetes regarding the use of HC as only carbon source. This kind of cultures is quite difficult to grow because HC do not mix well in the hydrophilic culture media which showed to be a great challenging for the obtainment of reproducible results. The use of the detergent dichloromethane was also assayed in the hope that it helped in the mixing of the hydrocarbons. However this detergent affected the growth of planctomycetes (Fig. 7) and it could not be used in other experiments.

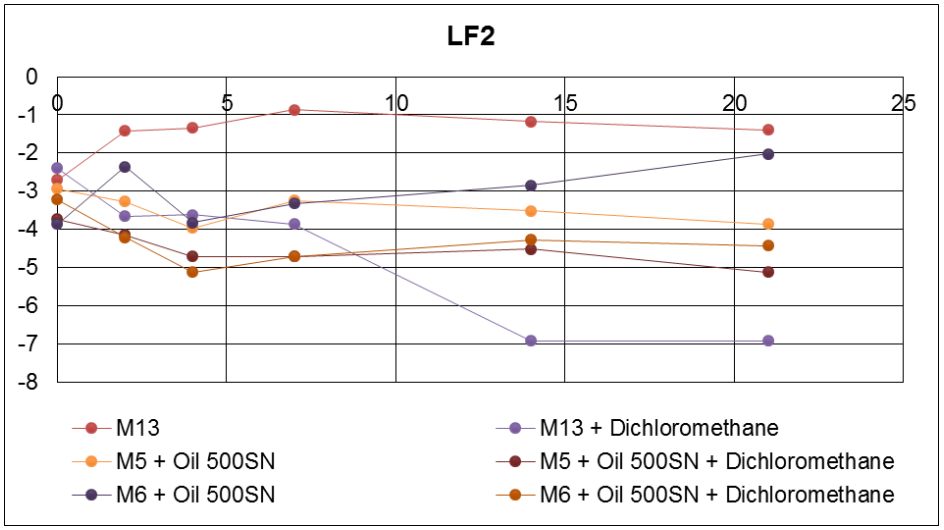

Figure 7 - Growth of strain LF2 in several media with different nitrogen and carbons source with addiction of dichloromethane. Growth of strain LF2 is clarity affected for addiction of Dichloromethane.

4.2 Planctomycetes acute assay in the presence of furfural

Furfural is an aldehyde of furan originated from a variety of agricultural by products like oat and wheat. Aiming at assessing the potential growth of planctomycetes in the presence of furfural, ten strains of planctomycetes, FC3, FC18, FF15, Gr7, LF2, MsF5, Pd1, SH1, UC8 and UC9, were assayed against this substance in an acute toxicity assay of 30 minutes exposure. The planctomycetes did not resist to any of concentrations tested. However, other bacteria were able to growth in this acute assay. These bacteria most probably were present in the furfural that was provided by Galp and future isolation and identification of these bacteria could be helpful for the waste treatment.

4.3 Assessment of Planctomycetes growth in the water from waste water treatment tank of the refinery

To assess if planctomycetes were able to growth in the presence of water from WWT tanks from Galp, *Rhodopirellula rubra* strains LF2 and *Rubinisphaera brasiliensis* strain Gr7 were grown in medium M1H supplemented with the waste water from different tank origins as source of carbon. Four different origins of waste water were tested. These waters were named accordingly to the localization in the circuit of the tank and the presence or "absence" of hydrocarbons (A1 – Dreno 26 with HC, A2 – Dreno 26, A3 – Dreno 50 A/B with HC and A4 – Dreno 50 A/B). However all the waters showed that HC are present in there composition. (Fig. 8).

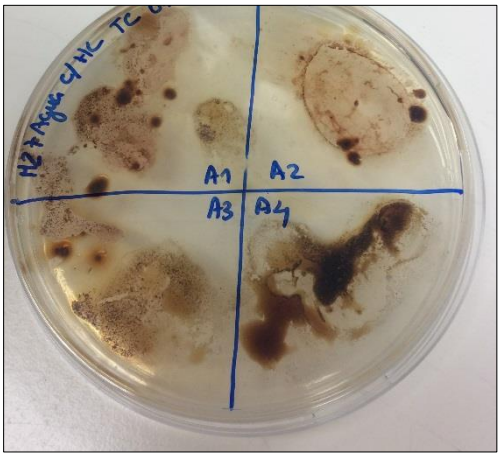

Figure 8 – Plate showing the growth of strain LF2 in the 4 different waste water provided.

After 7 to 10 days the characteristic growth of the two planctomycetes was evident (Figs. 8 and 9) and their affiliation confirmed by optical microscopy observation. Even though the composition of the waste water used was not known, hydrocarbons should be a main component. These results are indicative of the potential capacity of planctomycetes to be used as hydrocarbon degraders, but mores testes need to be done to prove that. We have to emphasize that this assay was only tested in solid medium.

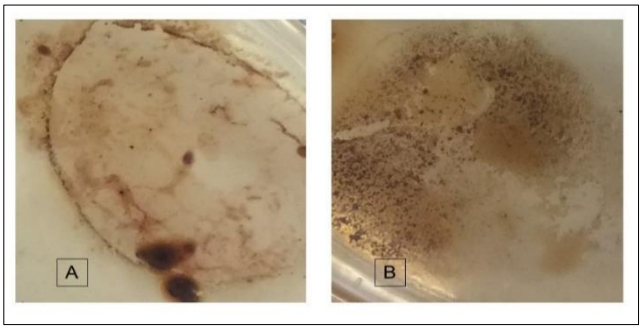

Figure 9 – Growth of strains tested in presence of waste water, from tanks of the refinery. A – strain LF2 and **B –** strain Gr7.

4.4 Isolation and characterization of microorganisms from the sludge of a waste water treatment tank of Galp refinery

4.4.1 Isolation process

Another challenged proposed by Galp was the biological characterization of sludge from the WWT tank. To cover the isolation of a wide range of microorganisms, several media were used: nutrient agar (NA), M13 and M1H this with different hydrocarbons as carbon source (crude oil and oils 150SN and 500SN). A total of 117 isolates were obtained in pure culture, with the highest number obtained in the freshwater medium NA, with 46 isolates (39%), followed by M13, a marine medium, with 35 isolates (30%). The attempts to isolate hydrocarbon degraders were done in M1H medium supplemented with crude oil, 150SN and 500SN oils. These media allowed a lower number of isolates, been the most efficient the one with crude oil with 18 isolates (15%) (Fig. 10). These isolation numbers are very high comparatively to the numbers obtained by other authors in comparable isolation experiments (from HC polluted environments) (Adebusoye,et al., 2007; Sawadogo et al., 2014; Shokrollahzadeh, et al., 2008).

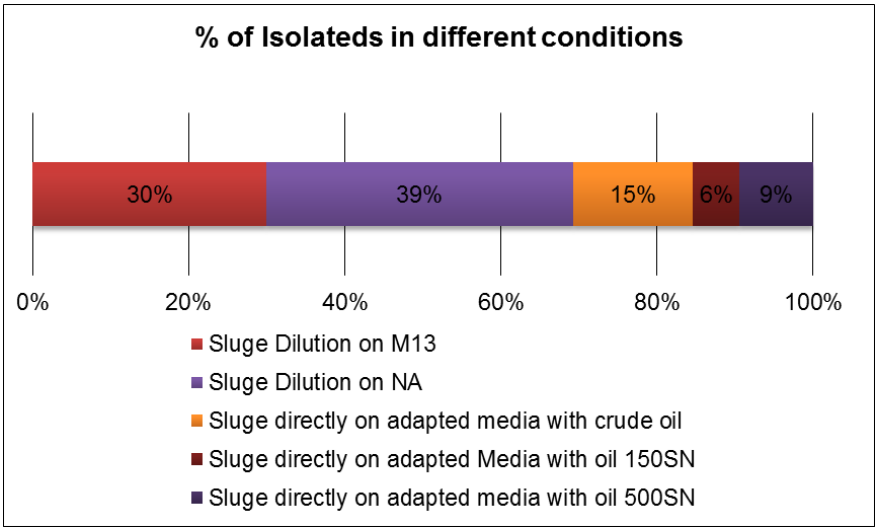

Figure 10 - Distribution of percentages of isolates obtained from each media. (Total of isolates= 117; NA isolates= 46; M607 isolates= 35; PET isolates= 18; 150SN= 7; 500SN= 11)

4.4.2 Identification and characterization of isolates

Although 117 isolates were initially obtained in pure culture, up-to-now only 96 were amplified for the 16S rRNA gene (Fig. 11) and only 74 were identified. Due to time constrains it was not possible to sequence all the isolated bacteria or obtain the full sequence (only forward or reverse sequences are available for some isolates). In this study, the most abundant phylum was *Firmicutes* with 31 isolates (42%), followed by

Proteobacteria with 30 isolates (40%), *Actinobacteria* with 12 isolates (16%) and *Bacteroidetes* with 1 isolate (1%) (Fig. 12).

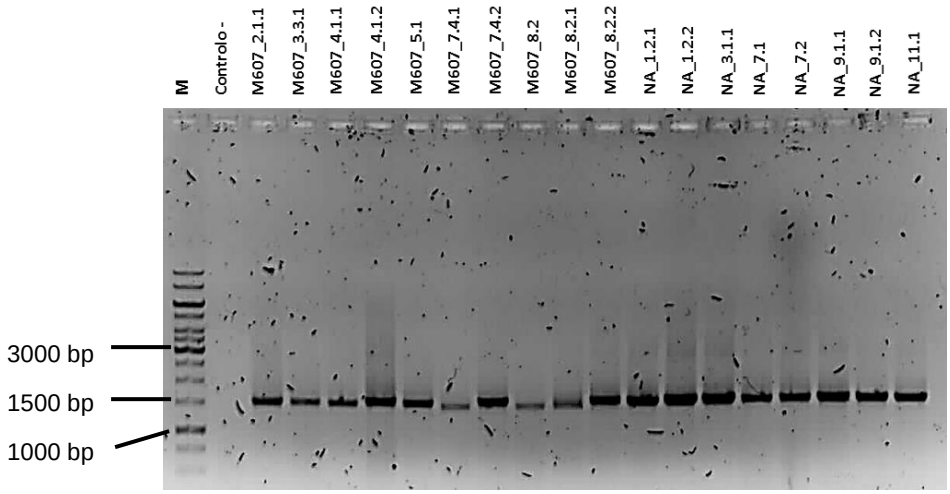

Figure 11 - Electrophoretic agarose gel evidencing the PCR products of 16S rRNA gene amplification from some isolates. M – Ladder (GeneRuler™ DNA Ladder Mix). The band shown have about 1500 bp. C- stands for Negative Control of the PCR reaction. For isolates designation see Table ZZ.

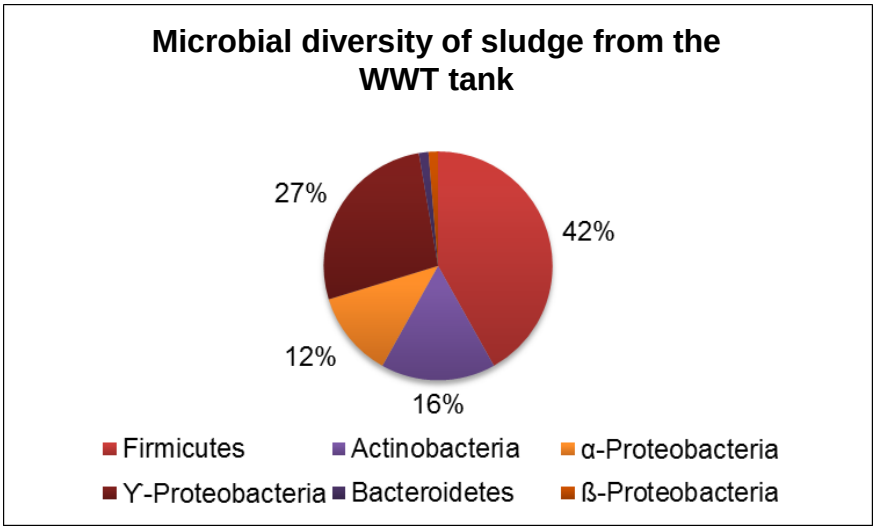

Figure 12 – Percentual distribution of isolates obtained from each different phylum. (Total number of isolates= 74; Firmicutes= 31; Proteobacteria= 30; Actinobacteria= 12; Bacteroidetes= 1)

Of the 74 identified isolates (Annex 1), some, apparently, are duplicates of the same organism and there are still others that present high similarity with more than one, thus, we obtained 10 different Operation Taxonomic units (OTUs) of *Firmicutes*, 4 different OTUs of *Actinobacteria*, 6 different OTUs of α-*Proteobacteria*, 9 different OTUs of *Y-Proteobacteria*, 1 OTU of *ß-Proteobacteria* and of *Bacteroidetes*. In total, we obtained 20 different genera from the sludge microbial community of Galp WWT tank. The highest diversity was observed in the *Proteobacteria* especially in the class

Gammaproteobacteria (Fig. 13) but the most abundant genera were *Bacillus* (17 isolates), followed by *Exiguobacterium* (10 isolates), *Arthrobacter* (8 isolates) and *Pseudomonas* (7 isolates) (Fig. 14).

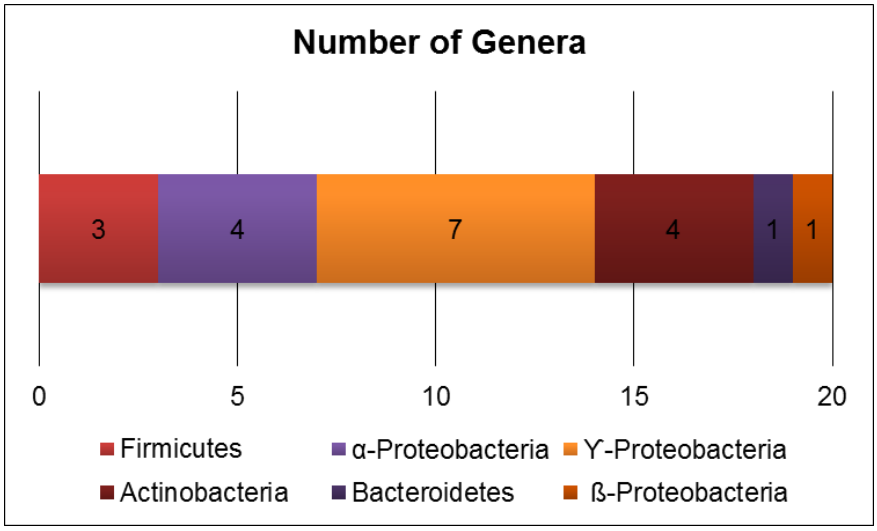

Figure 13 - Number of obtained isolates from each different genera. (Total number of genera= 20; Genera of *Firmicutes*= 3; Genera of *Proteobacteria*= 12; Genera of *Actinobacteria*= 4; Genera of *Bacteroidetes*= 1)

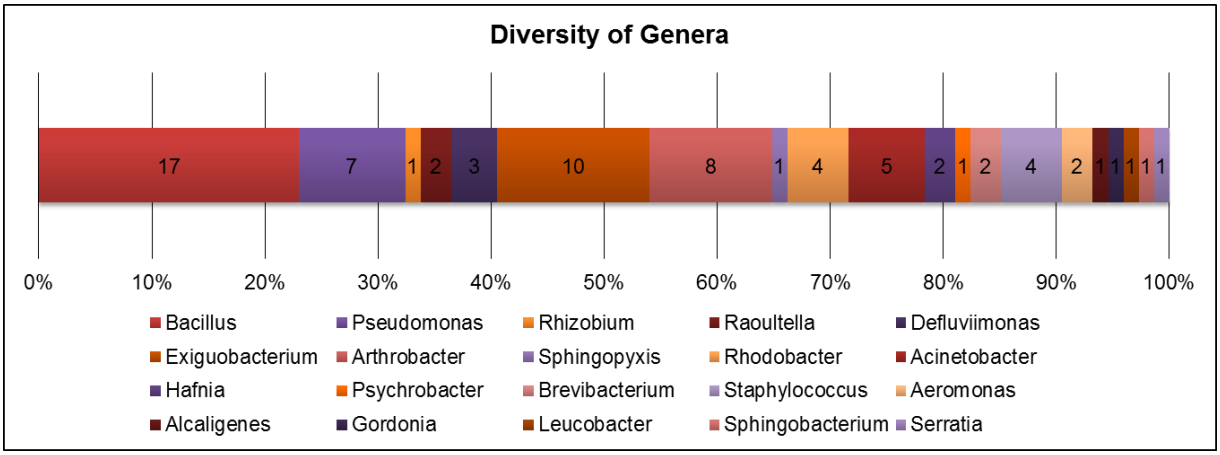

Figure 14 - Number of isolates of each genus. *Bacillus* – 17 isolates; *Pseudomonas* – 7 isolates; *Rhizobium* – 1 isolate; *Raoultella* – 2 isolates; *Defluviimonas* – 3 isolates; *Exiguobacterium* – 10 isolates; *Arthrobacter* – 8 isolates; *Sphingopyxis* – 1 isolate; *Rhodobacter* – 4 isolates; *Acinetobacter* – 5 isolates; *Hafnia* – 2 isolates; *Psychorobacter* – 1 isolate; *Brevibacterium* – 2 isolates; *Staphylococcus* – 4 isolates; *Aeromonas* – 2 isolates; *Alcaligenes* - 1 isolates; *Gordonia* – 1 isolate; *Leucobacter* – 1 isolate; *Sphingobacterium* – 1 isolate; *Serratia* – 1 isolate;

Differently from our results, Wagner et al. (2002) demonstrated that in activated sludge from WWT tanks the bacterial communities were dominated by the phylum *Proteobacteria* and *Bacteroidetes*. These different results can be explained by the media and methods use for the isolation and by the sludge composition. The isolation media NA and M13 provided a comparable number of genera (11 and 10 respectively), followed by the media using HC as a selection medium (Fig. 15).

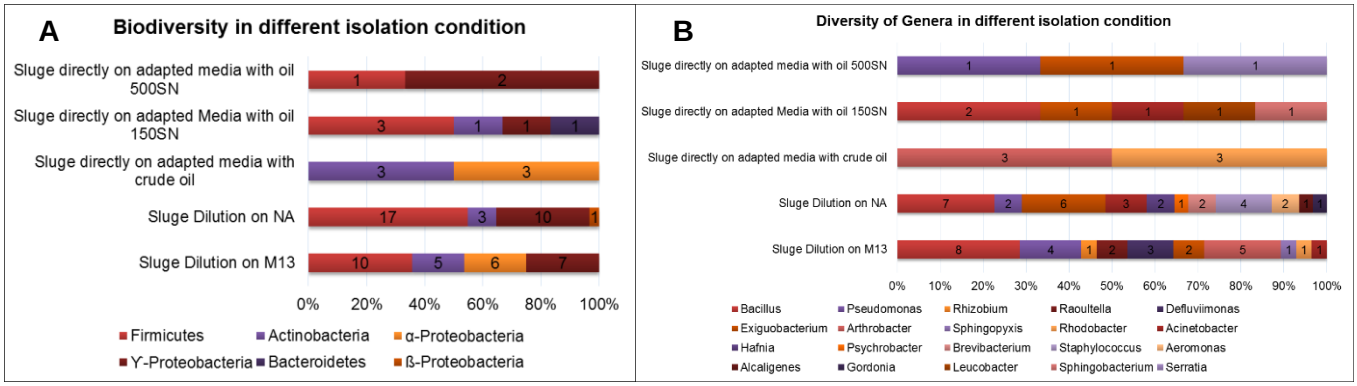

Figure 15 - Diversity of phylum (A) or genus (B) in obtained isolates with different isolation conditions. The medium that provided more phyla and genera was NA with 4 phyla and 11 genera, followed by M13 that provided 3 phyla and 10 genera, medium with oil 150SN with 4 phyla and 5 genera, medium with oil 500SN with 2 phyla and 3 genera and the medium with crude oil that provided just 2 phyla and 2 genera.

In this study, medium NA and medium with oil 150SN seemed to favour the phylum *Firmicutes* (Fig.15A) especially *Bacillus* (Fig.15B). Even not being the most abundant genus found in others studies, the genus was described as HC degrader and has been isolated from oil contaminated areas (Adebusoye, et al., 2007; Borzenkov, et al., 2006). Sass et al. (2008) isolated same strains from deep-sea hypersaline anoxic sediments, closely related to various *Bacillus* species like *B. subtilis*, *B. licheniforms*, *B. aquimaris* and *B. alcalophilus* all related to our isolates. They are able to growth using n-alkanes and aromatic compounds as the sole source of carbon (Sass et al., 2008). The genus *Exiguobacterium* was also described associated to HC polluted areas (Cai, et al., 2017), but our isolates are almost all related to *Exiguobacterium acetylicum,* a species described associated to root and rhizosphere soil (Zhang, et al., 2012). The genus *Staphylococcus* also obtained in this study, has been described to be associated to HC contaminated soils by Mujahid et al. (2015). Regarding the *Proteobacteria*, the only specimen of class *Betaproteobacteria*, *Alcaligenes faecalis,* was obtained on NA medium. Bharali et al. (2011) showed that *A. faecalis* isolated from soil samples of oil drilling sites of Assam (India) grow using crude oil as the sole source of carbon and energy.

On the other hand, medium M13, even presenting high number of *Firmicutes* especially *Bacillus*, also seemed to favour the phylum *Proteobacteria*, with the highest number on the *Gammaproteobacteria*, especially the *Pseudomonas* genus. One of the obtained isolate is relatively close to *Pseudomonas putida* strain FBHYA-B3, which was isolated from activated sludge from the aeration tank of an oil refinery complex (Farjadfard et al., 2010). In several *Pseudomonas* isolates specific enzyme systems such as cytochrome P450 alkane hydroxylases responsible for microbial HC degradation were detected (Van Beilen and Funhoff, 2007). As a matter of fact, the alkane hydroxylases of *Pseudomonas*

putida was characterized in great detail by Van Beilen and Funhoff (2007). *Pseudomonas* species and their closely related organisms have been the most extensively studied owing to their ability to degrade many different contaminants, in particular HC (Wackett, 2003). Another genus observed was *Raoultella*. The species most closed related with our isolate was the *Raoultella planticola* strain IL11 isolated from textile industry wastewater where it was involved in detoxification of aromatic compounds derived from dye-contaminated water (Kausar, 2015). The genus *Acinetobacter* were also obtained. The isolates assigned to this genus are often found in HC polluted environments (Ollivier and Magot, 2005) and our isolates are no exception, inclusive, two of our isolates that are closed related with two species also found in this type of environment, *Acinetobacter baumannii* from HC contaminated sites in India (Sarma, et al., 2004a) and *Acinetobacter venetianus*, that, according to Fondi et al. (2013), has genes involved in the metabolism of n-alkanes and in the resistance to toxic metals. The genus *Aeromonas* was also related with the isolates obtained. One of the isolates is closely related to *Aeromonas hydrophila.* This species was already identify in isolation from soil contaminated with crude oil and described as a diesel oil degrader by Kaczorek, et al. (2010). Also the genus *Psychrobacter*, for the same class, was already described associated to the diesel-degrading bacterial consortium (Ciric, et al., 2010) which was closely related to one of our isolates. *Serratia marcescens* is another species that is closely related to one of our isolates. This species was isolated from the root of *Wasabia japonica* (Li et al., 2014). The genus *Serratia* was described by Odokumal and Dickson (2003) as part of a bioremediation consortium isolated from crude oil polluted tropical rain forest soil. Still regarding *Gammaprobacteria*, two of the obtained isolates are closely related with genus *Hafnia*. The species closely related was *Hafnia alvei* that is a commensal of the animals guts (Li et al., 2014). The ability of this genus to degrade HC compounds appears to be an unusual feature. The medium supplemented with crude oil also seemed to favour the isolation of *Proteobacteria* specially *Alphaproteobacteria* of the genus *Rhodobacter*. The species closely related to some isolates was *R. sphaeroides*. This genus has not been yet related with HC degradation or HC pollution environments, but this is a genus that plays very important environmental roles because it possesses diverse abilities including metal reduction, nitrogen fixation, and the assimilation of carbon dioxide (Callister et al., 2006). Samanta et al. (2002) suggested that *R. sphaeroides* is a good candidate for bioremediation since it has several mechanisms of adaptation to different environments and compounds. As *R. sphaeroides*, the species *Defluviimonas denitrificans* has not been yet related to HC degradation or HC pollution environments. This species was isolated from the biofilter of

a marine aquaculture (Foesel, et al., 2011). Another genus closely related to our isolates was *Rhizobium*. *Rhizobium* species were already related to HC polluted environments (Kaiya et al., 2012a) and were also describe as able to utilized HC as the sole carbon and energy source (Kaiya et al., 2012b). Regarding *Alphaproteobacteria*, *Sphingopyxis* (or *Sphingomonas*) was also closely related to our isolate. This genus was already isolated from HC polluted environments (Leys et al., 2004).

The 3rd phylum provided by M13 medium was *Actinobacteria*, and especially seemed to favour the genus *Arthrobacter*. The genus *Arthrobacter* was already described as a HC degrader by several authors (Stevenson, 1967; Plotnikova et al., 2011) and also identified as closely related with same isolates from HC contaminated side in Hilo (USA) (Seo, et al., 2006). Another genus closely related to our isolates was *Brevibacterium*. Chaillan et al. (2004) identify biodegradation potential of some strains isolated from crude oil activity site and one of them was *Brevibacterium* species. Our isolates of genus *Gordonia* were closely related to several ones associated with HC polluted environments (Kummer, et al., 1999; Hernandez-Perez, et al., 2001; Xue et al., 2003; Borzenkov et al., 2006). The genus *Leucobacter*, also an Actinobacteria, was closed related to our isolates. This genus was also described as able to use HC as carbon and energy source and it has been isolated from abandoned industrial sites (Zocca, et al., 2004).

The media supplemented with 150SN allowed obtaining the only specimen for the phylum Bacteroidetes, the genus *Sphingobacterium*. This genus was related with HC contaminated sites by Owsianiak et al. (2009).

Many identified isolates showed a similarity of 100% to their closest cultivated relatives but specially one *Actinobacteria* (NA_15), one *Gammaproteobacteria* (150SN_1.4.1) and four *Alfaproteobacteria* (M607_7.3, PET_1.1.1, PET_1.2.1 and PET_1.4.3) were only 91.7%, 96.3%, 95.4%, 93.7%, 93.6% and 91.2%, respectively, similar to their closest relatives. These bacteria are potentially novel taxa that should be further characterized.

Figure 16 - Phylogenetic 16S rRNA gene tree was constructed using Geneious R10 tree builder, by choosing the Tamura-Nei model and the neighbor-joining method with a bootstrap resampling method considering 1000 replicates. The Archae Sulfolabus acidocaldarius was used as outgroup.

4.4 Summing up

The initial goal of this work was to explore the role of planctomycetes in the degradation of HC. For that we grew several planctomycetes in different media with HC as unique source of carbon and energy, as well, different inorganic nitrogen sources to potentiate their growth. The results of the growth in solid media were very promising, so we proceed with growth in liquid media. In this step of the work we found a lot of obstacles and the results were difficult to interpret. In the future more assays need to be done to confirm the importance of planctomycetes on degradation of HC compounds and their role on bioremediation process.

In an attempt to achieve some answers to the problems faced by the Galp Company we extended this work to other aims. One was to try to cope with the problems due to furfural that affects the biodiversity existing on sludge the wastewater tanks. Therefore, it was important to enrich the existing biodiversity with organisms that are resistant to this compound. So we try to asses if planctomycetes were able to grow after exposure to this compound. In an acute assay planctomycetes showed to be sensible to furfural. However, some microorganisms that could grow on this assay, might be organisms that they help solving the furfural. Future work needs to be done on the identification, characterization and application of these organisms.

Another aim was to try to improve the refinery wastewater treatment process with planctomycetes. So we assessed if planctomycetes were able to growth in the water from WWT tanks. As planctomycetes were able to growth in these waters as sole source of carbon and energy, they might be good candidates to enrich the biodiversity of tank sludge and to enhance the treatment of residues of the refinery. More studies need to be done.

A final aim of this work was to isolate and identify the diversity of sludge from a WWT tank for a better biological understanding of the process envisaging in the future to potentiate the waste treatment. 117 isolates were obtained of which 74 were identified. The number of isolates obtained was very high comparatively to the isolates obtained in comparable isolation experiments. In this study, the most abundant phylum was *Firmicutes* (42%), followed by *Proteobacteria* (40%) (especially *Gammaproteobacteria*), *Actinobacteria* (16%) and *Bacteroidetes* (1%).

5. Conclusions and Future perspectives

This work showed, through different approaches, the potential of Planctomycetes as hydrocarbon degraders which make them good candidates for the remediation of hydrocarbon polluted environments.

It also contributed to enlarge our knowledge on the microbial community associated with these habitats and allowed the isolation of strains that are novel taxa.

This work opened several future perspectives that we ought to perform in the future. These included the confirmation of the Planctomycetes growth in liquid media, the development of new assays in small scale for HC degradation, the characterization of the six strains that are novel new taxa and the isolation and characterization of furfural resistant bacteria. This work, performed in collaboration with the oil Company, may have a positive impact for the solution of problems faced by Galp.

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7. Annexes

Annex I

Table I – Results of the Blast in RDP database, performed with the obtained sequences of the 16S rRNA of the isolates**.**

Isolate	HQ%	RDP Identification	16S rRNA ref seq	s_ab score	Phylum	Class	Genera
M607_1.1	98.1	Pseudomonas putida; Pseudomonas fulva	AF094741/ DQ141541	1,000	Proteobacteria	Ɣ-Proteobacteria	Pseudomonas
M607_1.2.1	98.4	Pseudomonas putida; Pseudomonas fulva	AF094741/ DQ141541	1,000	Proteobacteria	Ɣ-Proteobacteria	Pseudomonas
M607_1.2.2 - F	55.3	Bacillus aquimaris; Bacillus vietnamensis	KJ577057 / JQ799104 / JX460828	0,944	Firmicutes	Bacilli	Bacillus
M607_1.2 - R	89.0	Bacillus alcalophilus; Bacillus aquimaris; Bacillus vietnamensis	EU231621 / FJ613316 / FJ188309	1,000	Firmicutes	Bacilli	Bacillus
M607_2.1.2 - F	72.1	Pseudomonas putida;	HQ166061	0,991	Proteobacteria	Ɣ-Proteobacteria	Pseudomonas
M607_2.1 - R	78.0	Bacillus aquimaris	KC335217	0,978	Firmicutes	Bacilli	Bacillus
M607_2.2	97.7	Pseudomonas putida; Pseudomonas plecoglossicida	AM411058 / EU594553	1,000	Proteobacteria	Ɣ-Proteobacteria	Pseudomonas
M607_3.1	98.2	Rhizobium radiobacter	AJ295683	0,992	Proteobacteria	α-Proteobacteria	Rhizobium
M607_3.2 - F	95.4	Raoultella planticola	KT361501	0,996	Proteobacteria	Ɣ-Proteobacteria	Raoultella
M607_3.3.1	88.5	Raoultella planticola	AF129444	0,993	Proteobacteria	Ɣ-Proteobacteria	Raoultella
M607_3.3.2	94.9	Defluviimonas denitrificans	AM403233	0,998	Proteobacteria	α-Proteobacteria	Defluviimonas
M607_4.1	97.7	Exiguobacterium acetylicum	JF703652	1,000	Firmicutes	Bacilli	Exiguobacterium
M607_4.1.1	98.4	Arthrobacter protophormiae; Arthrobacter soli (T); Arthrobacter bergerei	EU430095/ EF660748/ KP684096	1,000	Actinobacteria	Actinobacteria	Arthrobacter
M607_4.2	98.1	Arthrobacter bergerei; Arthrobacter soli	KP684096 / KP684095	0,998	Actinobacteria	Actinobacteria	Arthrobacter
M607_4.3 - R	97.6	Arthrobacter protophormiae; Arthrobacter soli (T)	EU430095/ EF660748	1,000	Actinobacteria	Actinobacteria	Arthrobacter
M607_5.1 - R	97.7	Arthrobacter protophormiae; Arthrobacter soli (T)	EU874451/ EF660748	1,000	Actinobacteria	Actinobacteria	Arthrobacter
M607_5.2	99.4	Arthrobacter protophormiae; Arthrobacter soli (T)	EU430095/ EF660748	0,995	Actinobacteria	Actinobacteria	Arthrobacter

M607_6.2	98.3	Exiguobacterium acetylicum	JF703652	0,996	Firmicutes	Bacilli	Exiguobacterium
M607_7.1 - R	94.8	Bacillus baekryungensis; Bacillus hwajinpoensis	AY505501 / JN208075	1,000	Firmicutes	Bacilli	Bacillus
M607_7.2.2	96.8	Defluviimonas denitrificans	AM403233	1,000	Proteobacteria	α-Proteobacteria	Defluviimonas
M607_7.2 - R	88.2	Sphingomonas adhaesiva; Sphingopyxis ummariensis (T); Sphingopyxis terrae; Sphingopyxis macrogoltabida;	FJ009391 / EF424391 / AB680910 / AB680906	1,000	Proteobacteria	α-Proteobacteria	Sphingopyxis
M607_7.3	99.6	Rhodobacter aestuarii	FN997618	0,954	Proteobacteria	α-Proteobacteria	Rhodobacter
M607_7.3.1	99.2	Bacillus baekryungensis	AY505501	1,000	Firmicutes	Bacilli	Bacillus
M607_7.4	98.9	Bacillus baekryungensis	AY505501	1,000	Firmicutes	Bacilli	Bacillus
M607_7.4.2	98.7	Bacillus baekryungensis	AY505502	0,998	Firmicutes	Bacilli	Bacillus
M607_8.1 - R	77.7	Acinetobacter baumannii	HQ645939	0,985	Proteobacteria	Ɣ-Proteobacteria	Acinetobacter
M607_8.2.2 *	98.2	Bacillus baekryungensis	AY505501	1,000	Firmicutes	Bacilli	Bacillus
M607_8.2 - R *	55.7	Defluviimonas denitrificans	AM403233	1,000	Proteobacteria	α-Proteobacteria	Defluviimonas
NA_1.1 *	96.2	Hafnia alvei	KP462723	1,000	Proteobacteria	Ɣ-Proteobacteria	Hafnia
NA_1.2	88.7	Psychrobacter alimentarius; Psychrobacter allis; Psychrobacter faecalis; Psychrobacter aquaticus; Psychrobacter frigidicola; Psychrobacter luti	JX514419 / JX122558 / JX122560 / JX122562 / JX122588 / JX122605	0,997	Proteobacteria	Ɣ-Proteobacteria	Psychrobacter
NA_1.2.1	98.6	Brevibacterium linens; Brevibacterium aureum	AY243345 / AY299092	1,000	Actinobacteria	Actinobacteria	Brevibacterium
NA_1.2.2	98.3	Hafnia alvei	KP462723	1,000	Proteobacteria	Ɣ-Proteobacteria	Hafnia
NA_2.1 *	98.7	Exiguobacterium acetylicum	KC166866	1,000	Firmicutes	Bacilli	Exiguobacterium
NA_2.2	99.9	Exiguobacterium acetylicum	KC166866 / KR186177	1,000	Firmicutes	Bacilli	Exiguobacterium
NA_3.1	98.0	Exiguobacterium acetylicum	JF703652	1,000	Firmicutes	Bacilli	Exiguobacterium
NA_3.1.1	97.0	Staphylococcus hominis subsp. Null	L37601	1,000	Firmicutes	Bacilli	Staphylococcus
NA_4	97.9	Bacillus cereus; Bacillus thuringiensis	AY224380 / Z84588	1,000	Firmicutes	Bacilli	Bacillus
NA_5.1	97.4	Acinetobacter junii	AM184300	1,000	Proteobacteria	Ɣ-Proteobacteria	Acinetobacter
NA_5.2	97.3	Aeromonas hydrophila subsp. Null	AY987754	1,000	Proteobacteria	Ɣ-Proteobacteria	Aeromonas
NA_7.1	98.3	Bacillus thuringiensis; Bacillus toyonensis	FJ772018 / KP406730	0,992	Firmicutes	Bacilli	Bacillus
NA_7.1.1	98.2	Bacillus thuringiensis; Bacillus toyonensis	FJ772018 / KM272775	0,998	Firmicutes	Bacilli	Bacillus
NA_7.2 - F	90.2	Bacillus thuringiensis	KT895850	0,979	Firmicutes	Bacilli	Bacillus

NA_9.1	98.0	Bacillus subtilis (T); Bacillus licheniformis	AJ276351 / AF391127	1,000	Firmicutes	Bacilli	Bacillus
NA_9.1.1 - F *	92.9	Bacillus subtilis; Bacillus tequilensis	KJ620421 / KC172053	1,000	Firmicutes	Bacilli	Bacillus
NA_9.1.2	97.9	Bacillus subtilis (T); Bacillus licheniformis	AJ276351 / AF391127	1,000	Firmicutes	Bacilli	Bacillus
NA_9.2 - R	96.1	Pseudomonas putida; Pseudomonas fulva	AF094741/ DQ141541	1,000	Proteobacteria	Ɣ-Proteobacteria	Pseudomonas
NA_10 - R	96.9	Acinetobacter baumannii; Acinetobacter calcoaceticus (T)	AY847284 / AJ888983	0,997	Proteobacteria	Ɣ-Proteobacteria	Acinetobacter
NA_11.1	98.8	Brevibacterium linens; Brevibacterium aureum	AY243345 / AY299092	1,000	Actinobacteria	Actinobacteria	Brevibacterium
NA_11.1.1 *	98.9	Exiguobacterium acetylicum	KC166866	1,000	Firmicutes	Bacilli	Exiguobacterium
NA_11.1.2	96.7	Exiguobacterium acetylicum	KC166866	1,000	Firmicutes	Bacilli	Exiguobacterium
NA_11.2	98.7	Exiguobacterium acetylicum	KC166866	1,000	Firmicutes	Bacilli	Exiguobacterium
NA_12.1	98.7	Staphylococcus saprophyticus subsp. Null	HQ407261	1,000	Firmicutes	Bacilli	Staphylococcus
NA_12.2	99.2	Staphylococcus saprophyticus subsp. Null	HQ407261	1,000	Firmicutes	Bacilli	Staphylococcus
NA_12.2.1	98.8	Staphylococcus saprophyticus subsp. Null	HQ407261	1,000	Firmicutes	Bacilli	Staphylococcus
NA_14	98.6	Alcaligenes faecalis	JF682513	1,000	Proteobacteria	ß-Proteobacteria	Alcaligenes
NA_15 - R *	83.4	Gordonia spumae; Gordonia australis	AJ251724 / DQ150570	0,917	Actinobacteria	Actinobacteria	Gordonia
NA_17	98.8	Acinetobacter sp. phenon 8; Acinetobacter junii	AJ633634 / AM184300	1,000	Proteobacteria	Ɣ-Proteobacteria	Acinetobacter
NA_18	98.3	Aeromonas hydrophila subsp. Null	AY987754	1,000	Proteobacteria	Ɣ-Proteobacteria	Aeromonas
NA_19 - R	97.5	Pseudomonas putida; Pseudomonas fulva	AF094741/ DQ141541	1,000	Proteobacteria	Ɣ-Proteobacteria	Pseudomonas
150SN_1.2 *	98.8	Bacillus aquimaris	KF933617	1,000	Firmicutes	Bacilli	Bacillus
150SN_1.2.1	97.8	Leucobacter aridicollis (T)	AJ781047	1,000	Actinobacteria	Actinobacteria	Leucobacter
150SN_1.3	96.9	Exiguobacterium arabatum; Exiguobacterium profundum	FM203124 / JN644510	0,996	Firmicutes	Bacilli	Exiguobacterium
150SN_1.4	97.3	Sphingobacterium mizutaii;	JF708889	0,997	Bacteroidetes	Sphingobacteriia	Sphingobacterium
150SN_1.4.1 - R	86.5	Acinetobacter beijerinckii; Acinetobacter venetianus VE-C3	HQ425646 / CM001772	0,963	Proteobacteria	Ɣ-Proteobacteria	Acinetobacter
150SN_1.4.2 - F	94.2	Bacillus aquimaris	KF933617	1,000	Firmicutes	Bacilli	Bacillus
500SN_1.1.2 - R	92.7	Pseudomonas putida; Pseudomonas fulva	AF094745/ DQ141541	0,981	Proteobacteria	Ɣ-Proteobacteria	Pseudomonas
500SN_1.3 - R	92.7	Serratia marcescens subsp. sakuensis	KC790285	0,997	Proteobacteria	Ɣ-Proteobacteria	Serratia

500SN_2.1	97.6	Exiguobacterium acetylicum	JF703658	1,000	Firmicutes	Bacilli	Exiguobacterium
PET_1.1.1 - R *	84.4	Rhodobacter sphaeroides	GU370089	0,937	Proteobacteria	α-Proteobacteria	Rhodobacter
PET_1.2.1 - R *	94.7	Rhodobacter sphaeroides	GU370089	0,936	Proteobacteria	α-Proteobacteria	Rhodobacter
PET_1.2.2 - R	95.4	Arthrobacter protophormiae; Arthrobacter soli (T)	EU874451/ EF660748	0,992	Actinobacteria	Actinobacteria	Arthrobacter
PET_1.3.1 - R	88.5	Arthrobacter protophormiae; Arthrobacter soli (T)	EU430095/ EF660748	0,988	Actinobacteria	Actinobacteria	Arthrobacter
PET_1.4.2 - R	98.1	Arthrobacter protophormiae; Arthrobacter soli (T)	EU874451/ EF660748	1,000	Actinobacteria	Actinobacteria	Arthrobacter
PET_1.4.3	99.7	Rhodobacter sphaeroides; Rhodobacter johrii	GU370089 / KJ776411	0,912	Proteobacteria	α-Proteobacteria	Rhodobacter